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Lipooligosaccharide Structures of Invasive and Carrier Isolates of *Neisseria meningitidis* Are Correlated with Pathogenicity and Carriage*[S]

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The degree of phosphorylation and phosphoethanolaminylation of lipid A on neisserial lipooligosaccharide (LOS), a major cell-surface antigen, can be correlated with inflammatory potential and the ability to induce immune tolerance *in vitro*. On the oligosaccharide of the LOS, the presence of phosphoethanolamine and sialic acid substituents can be correlated with *in vitro* serum resistance. In this study, we analyzed the structure of the LOS from 40 invasive isolates and 25 isolates from carriers of *Neisseria meningitidis* without disease. Invasive strains were classified as groups 1–3 that caused meningitis, septicemia without meningitis, and septicemia with meningitis, respectively. Intact LOS was analyzed by high resolution matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Prominent peaks for lipid A fragment ions with three phosphates and one phosphoethanolamine were detected in all LOS analyzed. LOS from groups 2 and 3 had less abundant ions for highly phosphorylated lipid A forms and induced less TNF-α in THP-1 monocytic cells compared with LOS from group 1. Lipid A from all invasive strains was hexaacylated, whereas lipid A of 6/25 carrier strains was pentaacylated. There were fewer *O*-acetyl groups and more phosphoethanolamine and sialic acid substitutions on the oligosaccharide from invasive compared with carrier isolates. Bioinformatic and genomic analysis of LOS biosynthetic genes indicated significant skewing to specific alleles, dependent on the disease outcome. Our results suggest that variable LOS structures have multifaceted effects on homeostatic innate immune responses that have critical impact on the pathophysiology of meningococcal infections.

Our previous studies of neisserial lipooligosaccharide (LOS)[2] and the human innate immune system have shown that the degree of phosphorylation of the lipid A component is correlated with the potential of the LOS to induce inflammation stimulated by innate immunity as revealed by cytokine induction in human monocytes *in vitro* and, in general, with the severity of infections (1–3). In the LOS of *Neisseria meningitidis*, the modification of lipid A with phosphoethanolamine (PEA) also has been shown to inhibit the bactericidal activity of cathepsin G within neutrophil extracellular traps (4) and to increase adhesion of the bacteria to human cells (5). Expression of PEA on the oligosaccharide (OS) of meningococcal LOS (Fig. 1), particularly in the *O*-3 position on heptose (Hep) II, or expression of sialic acid (Neu5Ac) have been shown to inhibit activation of complement via the classical and alternative pathways (6–9). In this study, we postulated that analysis of the structures of the LOS and the genomic diversity of genes for LOS biosynthesis would reveal differences associated with the severity of meningococcal infection and with carrier *versus* invasive strains of *N. meningitidis.*

To ascertain whether such differences exist, we characterized the structures of LOS from isolates from infected patients or from carriers of *N. meningitidis* that were collected during a double-blind randomized study of a serogroup B outer membrane meningococcal vaccine in teenagers in Norway in 1989–1991, and we analyzed the genomic diversity of genes involved in LOS biosynthesis of all meningococcal isolates sequenced to date. The LOS was purified, and the relative abundances of the lipid A phosphoforms were determined. We have previously shown that there can be losses of phosphoryl substituents from lipid A when the OS moiety has been cleaved by acidic hydrolysis of LOS and when the LOS is de-*O*-acylated to remove *O*-linked acyl groups (10). Thus, we analyzed the LOS intact using previously described methods on a Synapt G2 HDMS system for high mass resolution negative-ion matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) (10, 11). Some prompt fragmenta-

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[S] This article contains supplemental Table S1–S3.

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[2] The abbreviations used are: LOS, lipooligosaccharide; OS, oligosaccharide; PEA, phosphoethanolamine; TLR, toll-like receptor; miR-146a, microRNA-146a; P, phosphate; Hep, heptose; Kdo, 2-keto-3-deoxyoctulosonic acid; Neu5Ac, sialic acid; HexNAc, *N*-acetylhexosamine; TRAF, TNF receptor-associated factor; AOAH, acyloxyacyl hydrolase; HF, hydrogen fluoride.

tion of the LOS occurs during the MS analysis, enabling assignment of proposed compositions for the OS moieties as well as a comparison of the relative ion abundance of the lipid A phosphoforms.

Our results show that isolates producing LOS with the most highly inflammatory, most highly phosphorylated lipid A were more restricted to the CNS and had reduced ability to cause septicemia, which is correlated with higher case fatality rates. In addition, we found that the OS of the invasive isolates was more highly sialylated and phosphoethanolaminylated than OS from LOS of isolates from those individuals who did not become ill but only carried the bacteria. The results highlight the multifaceted and complex pathogenesis of meningococcal infections that uniquely occur in their human hosts.

Experimental Procedures

Bacterial Strains and LOS Preparation—Bacteria were 40 strains of *N. meningitidis* isolated from cerebrospinal fluids or blood cultures from patients (age 14–17 years) during the clinical trial with a group B outer membrane vesicle vaccine in Norway between years 1989 and 1991, and 25 strains were throat cultures of *N. meningitidis* isolated from carriers without disease during a phase II-6 clinical trial in Norway (12–14). Invasive strains were divided into three clinical diagnostic groups as follows: group 1 had meningitis, group 2 had septicemia without meningitis, and group 3 had septicemia with meningitis. Of the 40 invasive isolates, 29 were serogroup B, 11 were serogroup C, and 39 were categorized regarding clinical outcome (14). Serogroups B and C express polysaccharides composed of (α2→8)- and (α2→9)-linked sialic acid, respectively (15, 16). The serogroups of the carrier strains were more varied as follows: 7 B, 2 C, 1 W, 7 Y, and 8 NG strains (Tables S1 and S2). The dramatic differences in serogroup are in general accord with previous studies of nasopharyngeal carriage during outbreaks of infection in Norway that showed only a few carriers that harbored the prevailing outbreak strain (17, 18). The W and Y serogroup capsular polysaccharides are composed of alternating sialic acid moieties linked to D-galactose or D-glucose, respectively (19).

Preparation of Intact LOS for MALDI-TOF MS—Intact LOS samples were prepared for MS analysis using modifications of a method described previously (10). Briefly, purified LOS (4–10 mg/ml) was suspended in a methanol/water (1:3) solution with 5 mM EDTA, and an aliquot was desalted with a few cation exchange beads (Dowex 50WX8-200) that had been converted to the ammonium form. A spot containing a layer of matrix was formed by deposition of 1 or 2 drops (~1.0 μl each) of a solution composed of 2,4,6-trihydroxyacetophenone (200 mg/ml; Sigma) in methanol, with nitrocellulose transblot membrane (15 mg/ml; Bio-Rad) in acetone/isopropyl alcohol mixed in a 4:1 (v/v) ratio, within inscribed circles on the stainless steel sample plate. The nitrocellulose membrane was solubilized in the acetone/isopropyl alcohol solution (1:1, v/v) with vigorous vortexing. The desalted sample solution was mixed with 100 mM dibasic ammonium citrate (9:1, v/v), and 1.0 to 2.0 μl was deposited on top of spots of dried matrix. A second ultracentrifugation was performed to remove highly water-soluble capsular polysaccharide if MALDI-TOF MS analysis revealed few peaks at m/z >1000 or abundant peaks that differed by m/z 291, the residue mass of sialic acid, or a series of peaks that differed by m/z 453 that is in accord with the residue mass of sialic acid plus that of hexose (162 Da).

Hydrogen Fluoride (HF) Treatment of LOS—Native LOS was reacted with 48% aqueous HF to preferentially remove phosphoester moieties as described previously (1, 2). From 0.1 to 0.3 mg of LOS was placed in a 1.5-ml polypropylene tube, and cold 48% aqueous HF was added to make a 5–10 mg/ml solution, which then was allowed to react at 4 °C for 16–20 h. Excess HF was removed using a SpeedVac (Thermo Savant) with an in-line trap.

Negative-ion MALDI MS of Intact LOS—MALDI MS was performed on a Synapt G2 high definition MS (HDMS) system (Waters, Manchester, UK) with an orthogonal TOF mass analyzer in "sensitivity mode." The neodymium-doped yttrium aluminum garnet laser was operated with 355 nm at 200 Hz. Spectra were digitally smoothed and baseline-corrected using MassLynx software. The instrument was calibrated using the mass for the monoisotopic $(M - H)^-$ ions for bovine insulin at m/z 5728.5931, insulin B-chain at m/z 3492.6357, renin substrate at m/z 1756.9175, and angiotensin II at m/z 1044.5267. MALDI-TOF MS of intact LOS produces peaks for molecular and fragment ions $(M - H)^-$ and sodiated molecular and fragment ions $(M + Na - 2H)^-$ and $(M + 2Na - 3H)^-$, the latter despite use of ion-exchange beads to remove sodium, likely due to the presence of the acidic Kdo, phosphoryl, and Neu5Ac groups.

Quantitative Analysis of Phosphorylated Hexaacylated Lipid A Ions in Spectra of Intact LOS—We previously showed that the abundance of more highly phosphorylated lipid A fragment ion peaks in MALDI-TOF MS could be positively correlated with induction of inflammatory cytokines by the LOS by analysis of ion abundance ratios relative to the induction of TNF-α in THP-1 monocytic cells (2, 10). Here, we determined whether the ion abundances of more highly phosphorylated lipid A molecules from *N. meningitidis* causing disease or from carrier strains that did not cause disease were correlated with clinical outcomes of infection. This analysis was enabled by the observation of prompt Y-type reducing terminal fragment ions for the lipid A moieties of interest, including those that contained up to three phosphates and one PEA moiety in MALDI-TOF MS analysis. The MALDI spectra obtained on the Synapt G2 HDMS system were of high resolution enabling detection of monoisotopic peaks for the lipid A species. Areas of the common negative-ion peaks for hexaacylated lipid A were determined for 2–3 spectra for each LOS after using standard conditions for smoothing and correcting the baseline of the spectra using Mass Lynx Version 4.1 (Waters Corp.) software. Areas were included for the three most abundant isotopic peaks (M, M + 1, and M + 2) of the species given in Table 2. Average ratios were calculated of the ion abundance for peaks for hexaacylated lipid A containing three or four phosphates (3P or 4P), including PEA relative to the ion abundances for peaks for hexaacylated lipid A with 2P. The ion abundance for peaks at m/z 1737.1 was tabulated as four phosphoryl lipid A as these fragment ions arise from facile loss of $H_4P_2O_7$ from 3P PEA neisserial lipid A.

Compositional Analysis of the OS Moieties of LOS—We have shown that negative-ion MALDI-TOF MS using the thin layer matrix method of preparation of intact LOS produces molecule $(M - H)^-$ ions, as well as fragment ions for the lipid A and the OS moieties that together represent the entire LOS molecular. This method enables localization to the lipid A or the OS of substituents that are common to both moieties, such as PEA. The monosaccharide components that have been identified in OS from LOS from *N. meningitidis* and *N. gonorrhoeae* in analyses over the last three decades are as follows (20–26): 2-keto-3-deoxyoctulosonic acid (Kdo; nominal residue mass 220 Da); heptose (Hep; nominal residue mass 192); galactose and glucose (hexoses, Hexs; nominal residue mass 162); *N*-acetylglucosamine and *N*-acetylgalactosamine (*N*-acetylhexosamines; HexNAcs; nominal residue mass 203); *O*-acetate (nominal residue mass 42 Da); *O*-linked glycine (Gly; nominal residue mass 57); sialic acid (Neu5Ac; nominal residue mass 291); and PEA (nominal residue mass 123 Da). Two Kdo and two Hep were considered invariant components of the conserved core region. Substitution with Hex and HexNAc is invariant in the sense that these appear to always occur, but the number of each monosaccharide has been found to vary from 2 to 5 and 1 to 3, respectively. Sialic acid, *O*-acetylation, and *O*-glycination vary between 0 and 1 per LOS, and PEA varies from 0 and 2 per LOS. Because of the limited number of substituents that occur in LOS of *N. meningitidis*, the differences in the residue masses of the different substituents, and the observation of molecular ions and fragment ions that represent both the lipid A and the OS moieties in the same spectrum, we were able to propose OS compositions from careful analysis of the high quality, high resolution negative-ion MALDI spectra.

TNF-α Response of Human Monocytic Cells—The human monocytic leukemia cell line THP-1 was obtained from ATCC. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere and differentiated with 10 ng/ml phorbol myristate acetate (Sigma) for 18 h. Differentiated cells were seeded in 96-well plates at a concentration of 1×10^4 cells per well and treated for 18 h with LOS (100 ng/ml). The dose of LOS was based on our previous studies of their relative bioactivities (2, 27). Cell culture supernatants were assayed for TNF-α levels by ELISA as recommended by the manufacturer (human TNF-α ELISA Ready-SET-Go; eBioscience, San Diego, CA).

Bioinformatic Analysis of LOS Biosynthetic Genes—We used the PubMLST website developed by Jolley and Maiden (28), funded by the Wellcome Trust and sited at the University of Oxford, to analyze the allelic distribution of genes involved in LOS biosynthesis. We used the *Neisseria* spp. database to initiate all queries with the *N. meningitidis* limiter, and 32,202 entries were identified on August 20, 2015. Allelic profiles of LOS biosynthetic genes were identified as defined on the web site, and all subsequent analyses were performed on subsets of these data as indicated.

Allelic Assignments of LOS Biosynthetic Genes from Bacterial Strains—Genomic DNA was prepared using the MagNA Pure 96 system as recommended by the manufacturer (Roche Applied Science). DNA sequences encoding the *lot3*, *lptA*, and *lst* genes were determined from PCR amplicons generated using the following PCR primers: *lot3* (forward) ACACTGAGGTTTCCCGAGCTG and *lot3* (reverse) CAGCTCGGGAAACCTCAGTGT; *lptA* (forward) GCCCTGCTTTGCTCCGTTGC and *lptA* (reverse) TCCATATCGTTATACTCCGG; *lst* (forward) CGGTAACTGTCGGAATATCTGC and *lst* (reverse) ACGCAAAGCAATCAGAAATTCTC. DNA sequences of the amplicons were determined by Macrogen (Rockville, MD) using the same PCR primers. Occasionally, internal primers were designed from the derived sequence to generate complete sequences. For assignment of allele number, the *Neisseria* spp. database on the PubMLST website was queried with the gene sequence data for each strain.

Results

MALDI-TOF MS of Intact LOS from N. meningitidis—The negative-ion MALDI-TOF spectra of intact LOS contained peaks for molecular $(M - H)^-$ ions and for ions produced by prompt fragmentation occurring in the source that cleaved the labile bond between the non-reducing terminal *N*-acetylglucosamine moiety on the lipid A moiety and Kdo on the OS moiety (Fig. 1). Illustrative spectra are presented from the LOS of invasive disease-causing strain 14/90 in Fig. 2, *A–D*, and from carrier strains 190 and 419 in Figs. 2, *E–H*, and 3, *A–D*, respectively. Peaks in the spectra are labeled with *black*-colored font for $(M - H)^-$ molecular ions of intact LOS, *blue*-colored font for fragment ion peaks for the OS, and *red*-colored font for fragment ion peaks for the lipid A (Fig. 2). The high mass accuracy and high mass resolution of the spectra enabled baseline resolution of isotopic peaks as shown for the molecular ions (Figs. 2, *B* and *F*, and 3*B*). Labels give the *m*/*z* values for monoisotopic (^{12}C-containing) peaks. Listings of molecular ion and fragment ions and proposed compositions for prominent peaks observed in the spectra in Figs. 2 and 3 are presented in Table 1. The *m*/*z* values and proposed compositions for some of the prominent peaks observed in all of the spectra are presented in supplemental Tables S1 and S2, for the invasive and carrier strains, respectively.

Phosphoforms of Lipid A—Remarkably, the spectra of all of the strains were consistent with the predominant phosphoform of the lipid A having a single PEA and 3 phosphate groups. For the majority of the strains that produced hexaacylated LOS, the highest mass lipid A fragment ion was detected at *m*/*z* 1915.1 (Fig. 2, *C* and *G*) and is in accordance with a 3P PEA lipid A. In addition, lipid A fragment ion peaks were detected at *m*/*z* 1835.2, 1792.1, and 1712.1 that are consistent with 2P PEA, 3P, and 2P lipid A, respectively (Fig. 2, *C* and *G*). Peaks at *m*/*z* 1737.1 for a P PEA lipid A were detected that arise from facile loss of $H_4P_2O_7$ (178 Da) from 3P PEA lipid A (Table 2) as reported previously (29).

In the spectra from several strains, relatively very minor peaks were also detected at *m*/*z* 2038.1 in accordance with the expression of an LOS with 3P and 2PEA on lipid A (data not shown). However, clear evidence was lacking in the spectra for the expression of lipid A with a P PEA substituent on both the reducing and non-reducing termini that was reported previously (30), which would have been detected in hexaacylated lipid A at *m*/*z* 1958.1.

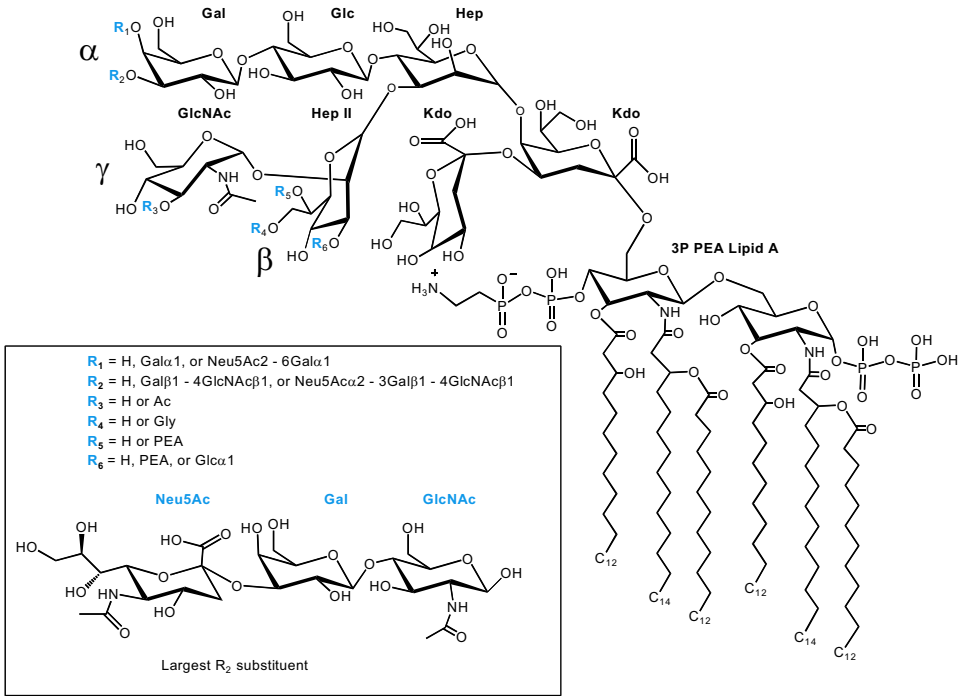


FIGURE 1. **Illustration of prototypical structures of LOS from *N. meningitidis*.** The prototypical lipid A as we describe in this report has 3P groups and 1 PEA as shown. Non-reducing terminal α, β, and γ chains on the oligosaccharide are illustrated.

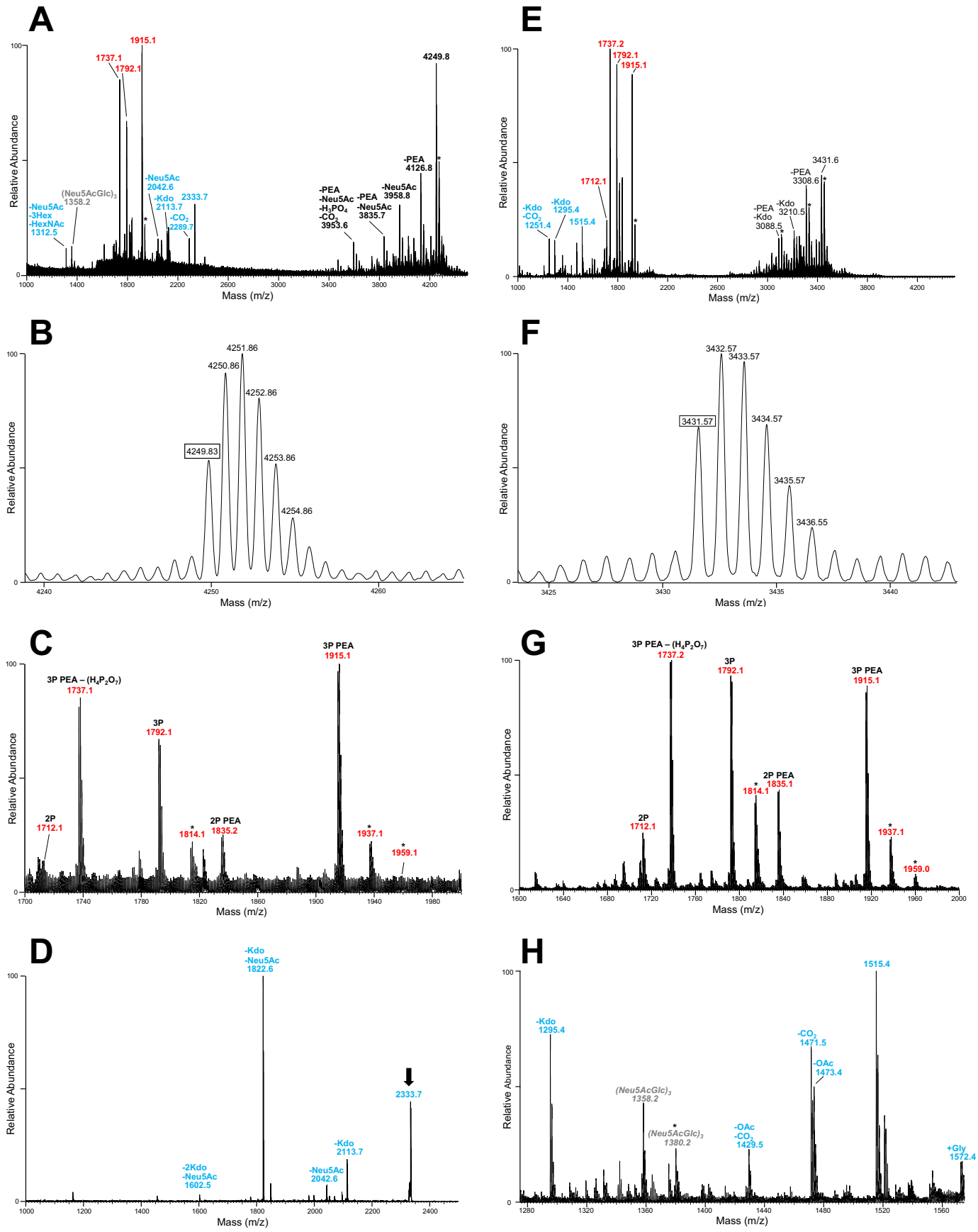
Relative Abundance of Lipid A Phosphoforms Correlates with TNF-α Induction—Quantification of ion abundance of the various phosphoforms of the lipid A enabled comparisons of the ratios of ion abundance of different states. As shown in Table 3*A*, analyzing the ion abundance of the predominant 3P PEA lipid A compared with that of the minor 2P lipid A species showed that there was a statistically significant greater degree of phosphorylation of the lipid A in disease group 1 compared with disease groups 2 and 3 when combined ($p = 0.038$).

The increase in phosphorylation in lipid A of disease group 1 was correlated with a statistically significantly greater induction of TNF-α expression by the LOS in THP-1 cells compared with the combination of disease groups 2 and 3 ($p < 0.05$) as shown in Fig. 4. LOS from group 1 induced higher level expression of TNF-α in the THP-1 cells and had a relatively greater proportion of the lipid A with four compared with two phosphoryl moieties relative to groups 2 and 3. Thus, the decreased *in vitro* inflammatory potential of LOS from the group 2 and 3 strains that caused septicemia reflected the decreased phosphorylation of lipid A. The level of lipid A phosphorylation and the average induction of TNF-α in THP-1 cells by the carrier strain LOS was intermediate to that of group 1 compared with groups 2 and 3 (Fig. 4, *A* and *B*).

Acylation of Lipid A—In all of the spectra from the disease and carrier isolates, molecular and fragment ion peaks were detected in accordance with the expression of the neisserial hexaacylated lipid A, except for the spectra of six carrier strains. In the spectra from these six strains, the prompt fragment ion peak for the 3P PEA lipid A was observed at *m*/*z* 1732.9 rather than 1915.1 as shown in Fig. 3, *A* and *C*, consistent with expression of a pentaacylated lipid A lacking a secondary lauroyl chain (182 Da). As in spectra of hexaacylated LOS, lipid A fragment ion peaks consistent with 2P PEA lipid A, 3P lipid A, and 2P lipid A were observed, but these were all lower by 182 Da being observed at *m*/*z* 1653.0, 1609.9, and 1530.0, respectively, rather than the peaks at *m*/*z* 1835.2, 1792.1 and 1712.1 observed for hexaacylated lipid A (Table 2). Peaks at *m*/*z* 1555.0 for a P PEA pentaacylated lipid A were detected likely due to loss of $H_4P_2O_7$ (178 Da) from 3P PEA lipid A similar to the prominent peaks observed at 1737.1 in the spectra of hexaacylated lipid A. As shown in Table 3B, the difference in acylation state between the invasive and carrier strains of *N. meningitidis* is statistically significant ($p = 0.0021$).

In the prototypical lipid A from *N. meningitidis,* there is a symmetrical pattern of acyloxyacylation, with *N*-linked 3-OH myristoyl groups on the 2- and 2′-positions of the reducing and the non-reducing terminal glucosamine moieties, respectively (Fig. 1). LpxL2 is the acyltransferase that transfers lauric acid to the 3-OH of the myristoyl moiety *N*-linked to the 2-position of the reducing terminal glucosamine. Acylation by a different acyltransferase, LpxL1, transfers lauric acid to the 3-OH of the myristoyl moiety *N*-linked to the 2′-position of the non-reducing terminal glucosamine, and apparently it is dependent on the presence of the lauroyl moiety transferred by LpxL2. Insertional inactivation of *lpxL2* led to the formation of a major lipid A species that lacked both acyloxyacyl groups (31). Significant peaks consistent with a tetraacylated lipid A such as a tetraacylated 3P PEA lipid A that would occur at *m*/*z* 1551, for example, were not observed in the spectra of the carrier strains with pentaacylated lipid A as illustrated in Fig. 3, *A* and *C*, indicating that the LOS was the product of an *lpxL1* mutant strain. Interestingly, all of the carrier strains with pentaacylated lipid A were of serogroup Y with the LOS having negligible ability to induce TNF-α expression in THP-1 cells (data not shown).

OS Compositions—A number of groups, including our own, have studied the structure of the OS from *N. meningitidis* (1, 23,


A
1915.1
1737.1
1792.1
4249.8
-PEA
4126.8
-Neu5Ac
2042.6
-Kdo
2113.7
2333.7
-CO2
2289.7
(Neu5AcGlc)3
1358.2
-Neu5Ac
-3Hex
-HexNAc
1312.5
-PEA
-Neu5Ac
-H3PO4
-CO2
3953.6
-PEA
-Neu5Ac
3835.7
-Neu5Ac
3958.8
Relative Abundance
Mass (m/z)
B
4251.86
4250.86
4252.86
4249.83
4253.86
4254.86
Relative Abundance
Mass (m/z)
C
3P PEA – (H4P2O7)
1737.1
3P PEA
1915.1
3P
1792.1
2P
1712.1
2P PEA
1835.2
1814.1
1937.1
1959.1
Relative Abundance
Mass (m/z)
D
-Kdo
-Neu5Ac
1822.6
2333.7
-Kdo
2113.7
-2Kdo
-Neu5Ac
1602.5
-Neu5Ac
2042.6
Relative Abundance
Mass (m/z)
E
1737.2
1792.1
1915.1
1712.1
-Kdo
-CO2
1251.4
-Kdo
1295.4
1515.4
3431.6
-PEA
3308.6
-PEA
-Kdo
3088.5
-Kdo
3210.5
Relative Abundance
Mass (m/z)
F
3432.57
3433.57
3431.57
3434.57
3435.57
3436.55
Relative Abundance
Mass (m/z)
G
3P PEA – (H4P2O7)
1737.2
3P
1792.1
3P PEA
1915.1
2P PEA
1835.1
1814.1
2P
1712.1
1937.1
1959.0
Relative Abundance
Mass (m/z)
H
1515.4
-Kdo
1295.4
-CO2
1471.5
-OAc
1473.4
(Neu5AcGlc)3
1358.2
(Neu5AcGlc)3
1380.2
-OAc
-CO2
1429.5
+Gly
1572.4
Relative Abundance
Mass (m/z)

24, 26, 32, 33). Compositions for the OS were assigned based on peaks we detected, the previously reported structures, and using the following potential components: 2 Kdo, 2 Hep, 1 or more Hex, and 1 or more HexNAc, 0–1 Neu5Ac, 0–1 *O*-Ac, 0–1 *O*-Gly, and 0–2 PEA. Calculation of the masses for combinations of these groups enabled assignment of OS compositions consistent with peaks observed in the mass spectra for the 65 strains (supplemental Tables S1 and S2).

Molecular and fragment ion peaks observed in the spectra presented in Figs. 2 and 3 are illustrative. The identity of the OS fragment ions shown in *blue*-colored font in Figs. 2, *A* and *E*, and 3*A* could be easily confirmed by noting that addition of the fragment ion mass to the neutral mass of the largest lipid A fragment ion equaled the mass of intact LOS. Thus, for example in Fig. 2*A*, the OS fragment ion peak at *m*/*z* 2333.7 plus the neutral mass of lipid A, 1916.1 Da, is equal to the prominent peak for intact LOS molecular ions at *m*/*z* 4249.8. Similarly, in Fig. 2*E* the OS peak at *m*/*z* 1515.4 plus the neutral lipid A mass of 1916.1 is equal to *m*/*z* 3431.6, and in Fig. 3*A*, the OS fragment ion peak at *m*/*z* 2210.7 plus the highest neutral mass of the lipid A, which is 1733.9 Da, is equal to the apparent molecular ion at *m*/*z* 3944.7.

Prompt fragmentation of labile groups from the OS moieties producing characteristic losses of Kdo (220 Da), CO_2 (44 Da) from Kdo, and Neu5Ac (291 Da) as observed previously, aided in confirming the identity of OS fragment ion peaks (10). In addition, peaks arising from losses of 42 Da from molecular and OS fragment ions were often observed, consistent with the presence of *O*-acetate as reported previously on LOS from some strains of *N. meningitidis* (10, 25). Molecular and OS fragment ion peaks indicative of the presence of glycine (Gly; 57 Da) were observed in LOS from five of the patient strains and 1 of the carrier strains as seen in Fig. 2*H* at *m*/*z* 1572.4 that differs by *m*/*z* 57 from the OS fragment ion peak *m*/*z* 1515.4. This finding is consistent with previous reports of the presence of Gly in the LOS of *N. meningitidis* (10, 34).

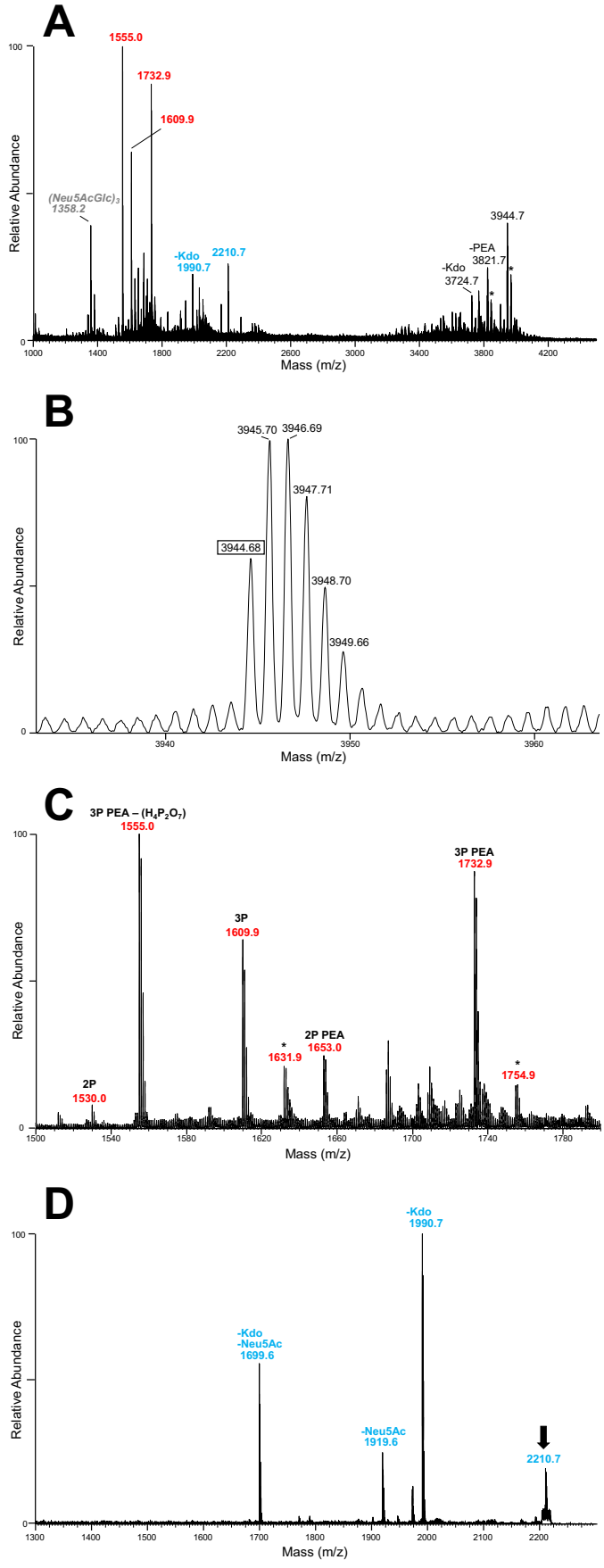
Fragment ion peaks in accord with the loss of PEA from the molecular ions could be observed clearly in the spectra of LOS from disease strain 014/90 at *m*/*z* 4126.8 in Fig. 2*A* and from carrier strain 190 at *m*/*z* 3308.6 in Fig. 2*E*. In the spectrum of the LOS from carrier strain 419 that has an *lpxL1* mutation, a fragment ion peak consistent with the loss of PEA from the molecular ions could be seen at *m*/*z* 3821.7. The relative intensities of these three peaks compared with the intensity of the peaks for the intact molecular ions are comparable, although the OS of strain 419 lacks PEA substitution. Thus, the data indicate that most of the molecular ions lacking one PEA differ due to the lack or loss of PEA on lipid A rather than the OS portion of the LOS.

In some instances, analysis by MS/MS was useful in confirming OS compositions. The prompt OS fragment ion peaks formed in the source observed at *m*/*z* 2333.7 and 2210.7 in Figs. 2*D* and 3*D*, respectively, were collisionally activated with argon gas to produce fragment ions for loss of Kdo (−220 Da) and loss of Neu5Ac (−291 Da) confirming the sialylation of the OS.

The majority of the strains had two HexNAc substituents with either three or four Hex moieties with or without Neu5Ac substitution (supplemental Tables S1 and S2). These compositions are in accord with the expression of lacto-*N*-neotetraose (Galβ1–4GlcNAcβ1–3Galβ1–4Glc) that is attached to C4 of HepI in the absence or presence of an additional α1-linked Glc on C3 of HepII as reported previously (35, 36). In addition, there were three strains (062/90, 259, and 701) in which the OS composition had a single HexNAc and three Hex with Neu5Ac in accordance with the structure previously reported by Wakarchuk *et al.* (32) that had a Galα1–4Galβ1–4Glc (Pk human blood group trisaccharide) branch and an α2–6-linked Neu5Ac moiety. The composition of the LOS from four strains (002/89, 097/90, 145/91, and 190) had only two Hex with a single HexNAc and they were not sialylated, consistent with the presence of a lactose moiety, Galβ1–4Glc, as reported previously (24, 37).

Analyses of LOS from four of the invasive strains (046/89, 162/89, 014/90, and 032/91) and one of the carrier strains (strain 190) revealed peaks for intact molecular or OS fragment ions that had *m*/*z* values that were greater by 80 Da than the major component detected. Specific examples are peaks at *m*/*z* 2413.714 in the spectrum of LOS from the invasive strain 014/90 and at *m*/*z* 1595.410 in the spectrum of LOS of the carrier strain 190 (Table 1). The 80-Da difference could be due to an additional P (exact mass 79.9663 Da) or it could be due to an additional HexNAc moiety (exact mass 203.0794 Da) plus the loss of a PEA (−123.0085 Da) that would present an additional 80.0709 Da. The mass difference between these two entities on the OS or intact LOS ions would be only ~60 and 30 parts per million (ppm), respectively. Neither of the potential compositions (OS with three HexNAcs or with a P substituent without ethanolamine) had been reported to our knowledge. All three of the invasive strains in question that apparently had either three HexNAc moieties or a P substituent also simultaneously expressed Neu5Ac, which primarily has been found to be linked (Neu5Acα2–3) to the terminal Gal of lacto-*N*-neotetraose. An OS with three HexNAc moieties has been reported in gonococcal LOS where a non-reducing terminal HexNAc moiety is attached (GalNAcβ1–3) to the terminal Gal of lacto-*N*-neotetraose and inhibits antibody binding and contributes to serum resistance (38, 39). Analyses of exact masses for the molecular and fragment ions revealed that these were more in accordance with the presence of a P ($\leq\Delta$15 ppm) and less so with the presence of an additional HexNAc with the loss of a PEA ($\geq\Delta$30 ppm).

FIGURE 2. *A–H,* high resolution negative-ion MALDI MS of intact LOS from *N. meningitidis* invasive strain 14/90 (*A–D*) that caused meningitis and septicemia and from 190, a carrier strain of *N. meningitidis* that did not cause disease (*E–H*). The $(M - H)^-$ peaks for the intact LOS are annotated in *black font*, whereas peaks for prompt fragment ions containing the lipid A moiety are labeled in *red*, and those of the oligosaccharide are in *blue*. *Asterisks* indicate peaks for sodiated species. Expansions of peaks are for the intact LOS molecular ions. *B* and *F,* illustrate the high mass resolution. An MS/MS spectra of the largest oligosaccharide fragment ion (indicated by an *arrow*) indicates the LOS contain sialic acid (Neu5Ac; *D*). An expansion of the spectrum of the OS from 190 is shown (*H*), which indicates that the OS lacks Neu5Ac but is glycinated (*Gly*). Some reports indicate that the phosphorylation of *N. meningitidis* lipid A is predominantly 2P 2PEA, but lipid A of LOS from the Norwegian isolates was invariably 3P PEA, with a minor amount of lipid A with 3P 2PEA detected in spectra of few strains.



To obtain further evidence of OS phosphoryl moieties, LOS from invasive strains 162/89 and 032/91, in which peaks for an additional 80 Da were observed in negative-ion spectra, were treated with aqueous HF to remove phosphoesters from the LOS. The spectra obtained produced abundant ions for molecules in which all P and PEA moieties had been cleaved from the LOS and no evidence suggesting the presence of an additional HexNAc (data not shown). Thus, overall the data support our assignment of these peaks as indicative of OS phosphoryl moieties.

Differences in OS Structures of LOS between Invasive and Carrier Strains—The mass spectra enabled determination of the apparent compositions of the OS from the 65 strains. Therefore, we analyzed the relationship between the composition of the OS and the pathogenicity of the bacteria according to clinical classification or carrier status. We found that there was a statistically significant increase in the expression of PEA on the OS from the invasive strains compared with OS of LOS from the carrier strains (Table 4A; $p = 0.010$). Differences between the groups of invasive strains in substitution of the OS with PEA, however, were not of statistical significance.

There also were differences between the invasive and carrier strains in the extent of *O*-acetylation and sialylation of the OS. The OS from 24 of the 25 (96%) carrier strains were found to have an *O*-acetate based on the analysis of the composition and observation of diagnostic losses of 42 Da, whereas only 60% (24 of 40) of the invasive strains expressed *O*-acetate, which was a statistically significant difference (Table 4*B*; $p = 0.0035$). The *O*-acetate of meningococcal LOS has been found on the C3 of the GlcNAc linked to the second Hep as shown in Fig. 1. Sialylation of the OS was observed in LOS from 37 out of 40 invasive strains (92.5%), whereas OS sialylation was found on statistically significantly fewer (18 of 25; 72%) carrier strains (Table 4C; $p = 0.0365$).

Genomic and Allelic Differences in LOS Biosynthetic Genes—To lend support for our conclusions of correlations between LOS structure and disease outcome, we analyzed the sequence diversity of LOS biosynthetic genes in a very large database of meningococcal genomes (32,202 genomes) found at the PubMLST website. The average number of unique alleles for the seven genes used for multilocus sequence typing was 529 alleles/gene (data not shown). The data shown in Table 5 demonstrate that the allelic diversity of genes involved in LOS biosynthesis and assembly is much more restricted, suggesting that these genes are under some form of selective pressure to limit LOS structural diversity.

We next analyzed the allelic distribution of two key genes in LOS biosynthesis and assembly, *lst* and *lptA*. The data pre-

FIGURE 3. *A–D,* high resolution negative-ion MALDI MS of intact LOS from *N. meningitidis* carrier strain 419. As in Fig. 2, $(M - H)^-$ peaks for the intact LOS are annotated in *black font*; those for prompt fragment ions for the OS are labeled in *blue*; and those of the lipid A are in *red*. *Asterisks* indicate sodiated peaks. The expansion of the peaks for the intact LOS molecular ions (*B*) illustrates the high mass resolution. The highest mass lipid A fragment ion in the spectra of the carrier strain observed at *m/z* 1732.9 differs by 182 Da from the virtually invariant lipid A fragment ion peak at *m/z* 1915.1 for a hexaacylated 3P PEA lipid A observed in the spectra of the disease-causing bacteria. The spectra of the carrier strain are consistent with the presence of a pentaacylated lipid A with 3P and 1 PEA due to *LpxL1* mutation.

TABLE 1
Proposed compositions for LOS peaks in spectra shown

Strain	Group	Diagnosis	$(M - H)^-$	Derived composition	Calculated $(M - H)^-$	Δ ppm
014/90	C	Septicemia	4249.858	TPHLA[a] PEA; 2 Hep, 2 Kdo, 2 HexNAc, 4 Hex, PEA, OAc, SA	4249.820	+8.89
			4126.815	TPHLA; 2 Hep, 2 Kdo, 2 HexNAc, 4 Hex, PEA, OAc, SA	4126.812	+0.70
			3958.736	TPHLA PEA; 2 Hep, 2 Kdo, 2 HexNAc, 4 Hex, PEA, OAc	3958.725	+2.88
			2413.714	2 Hep, 2 Kdo, 2 HexNAc, 4 Hex, PEA, OAc, SA, P	2413.681	+13.67
			2333.739	2 Hep, 2 Kdo, 2 HexNAc, 4 Hex, PEA, OAc, SA	2333.720	+8.06
			2113.665	2 Hep, Kdo, 2 HexNAc, 4 Hex, PEA, OAc, SA	2113.662	+1.37
			2042.634	2 Hep, 2 Kdo, 2 HexNAc, 4 Hex, PEA, OAc	2042.625	+4.55
			1915.091	TPHLA PEA	1915.092	−0.57
			1792.088	TPHLA	1792.084	+2.51
			1737.161	TPHLA PEA - ($H_4P_2O_7$)	1737.149	+6.79
190	NG	Carrier	3431.568	TPHLA PEA; 2 Hep, 2 Kdo, HexNAc, 2 Hex, PEA, OAc	3431.540	+8.28
			3308.559	TPHLA; 2 Hep, 2 Kdo, HexNAc, 2 Hex, PEA, OAc	3308.531	+8.43
			1915.109	TPHLA PEA	1915.092	+8.93
			1792.089	TPHLA	1792.084	+2.62
			1737.165	TPHLA PEA-($H_4P_2O_7$)	1737.149	+8.87
			1712.126	DPHLA[b]	1712.118	+4.73
			1595.410	2 Hep, 2 Kdo, HexNAc, 2 Hex, PEA, OAc, P	1595.406	+2.82
			1572.461	2 Hep, 2 Kdo, HexNAc, 2 Hex, PEA, OAc, Gly	1572.461	0.00
			1515.440	2 Hep, 2 Kdo, HexNAc, 2 Hex, PEA, OAc	1515.434	+3.96
			1295.376	2 Hep, Kdo, HexNAc, 2 Hex, PEA, OAc	1295.381	−4.48
419	Y	Carrier	3944.680	TPPLA[c] PEA; 2 Hep, 2 Kdo, 2 HexNAc, 4 Hex, OAc, SA	3944.645	+8.87
			3902.686	TPPLA PEA; 2 Hep, 2 Kdo, 2 HexNAc, 4 Hex, SA	3902.634	+13.32
			3821.647	TPPLA; 2 Hep, 2 Kdo, 2 HexNAc, 4 Hex, OAc, SA	3821.636	+2.88
			2210.719	2 Hep, 2 Kdo, 2 HexNAc, 4 Hex, OAc, SA	2210.712	+3.17
			2168.747	2 Hep, 2 Kdo, 2 HexNAc, 4 Hex, SA	2168.700	+21.67
			1732.930	TPPLA PEA	1732.925	+2.89
			1609.935	TPPLA	1609.917	+11.18
			1554.996	TPPLA PEA-($H_4P_2O_7$)	1554.982	+9.00

[a] TPHLA is triphosphoryl hexaacylated lipid A.
[b] DPHLA is diphosphoryl hexaacylated lipid A.
[c] TPPLA is triphosphoryl pentaacylated lipid A.

TABLE 2
Phosphorylated hexaacylated lipid A-negative ions

Phosphorylated lipid A moiety	Total phosphates	Monoisotopic peaks
P2-H	2	1712.2
P3 PEA-($H_4P_2O_7$)	4	1737.2
P3-H	3	1792.1
P3–2H + Na	3	1814.1
P2 PEA-H	3	1835.2
P3 PEA-H	4	1915.1
P3 PEA-2H + Na	4	1937.1
P3 PEA-3H + 2Na	4	1959.1

TABLE 3
Ion abundance ratios for phosphorylated (3P PEA/2P) hexaacylated LA (A) and expression of hexaacylated LA (B)
Data were compared with the average ratio of ion abundances for group 1.

A

Disease Group	n	Ratio 4P/2P	SD	Significance
1	19	10.798	4.943	---
2	12	7.680	3.223	NS
3	8	7.582	2.923	NS
2 & 3	20	7.641	2.951	$p = 0.038$
Carriers	19	8.952	3.462	NS

B

Disease Group	n	+ Hexaacylation	Significance
1, 2 & 3	40	40	---
Carriers	25	19	$p = 0.0021$

sented in Table 6 indicate that for *lst*, in carrier strains, there were three predominant alleles, 1, 3, and 15. In the disease isolates, although allele 1 was found in a significant proportion of the isolates, alleles 3 and 15 were found in only a small subset of these strains, with other alleles predominating, depending on the nature of the infection. This same type of genomic skewing was seen for *lptA,* where alleles 6 and 9 predominate in carrier strains, but in disease isolates, alleles 45 and 11 are predominantly represented.

Given the allelic skewing for *lst* and *lptA* when comparing disease and carrier strains in the genomic database, we assessed the allelic diversity of *lst*, *lptA,* and *lot3* in disease and carrier strains in this study. As shown in supplemental Table S3, allelic analyses revealed a predominance of alleles for all three genes from the disease isolates that were not found in the carrier strains, indicating the strains reflected the allelic diversity of these genes in disease and carrier isolates from the larger genomic database.

For *lst*, allele 2 was represented in three of seven disease strains but not in the nine carrier strains, whereas allele 16 was found in two carrier strains but not in disease isolates. Of interest is that disease strains 002/89 and 097/90 have an intact *lst* but do not sialylate their OS due to expression of a lactosyl α-chain, which is not an acceptor for sialic acid.

A similar pattern of allelic skewing exists for *lptA* with alleles 1 and 2 predominating in disease strains but not present in carrier isolates, and for *lot3* in which allele 94 was present in three of seven disease strains but not in the nine carrier strains examined, and allele 46 was present in three of nine carrier strains but not in the disease isolates. Notably for *lot3*, the three disease strains with allele 94 did not express *O*-acetate on the OS suggesting that this *lot3* allele encodes for a non-functional gene.

Discussion

We have previously shown that the degree of phosphorylation and phosphoethanolaminylation of lipid A on neisserial LOS can be correlated with inflammatory potential and ability to induce immune tolerance *in vitro* (1–3, 27). In this study, we

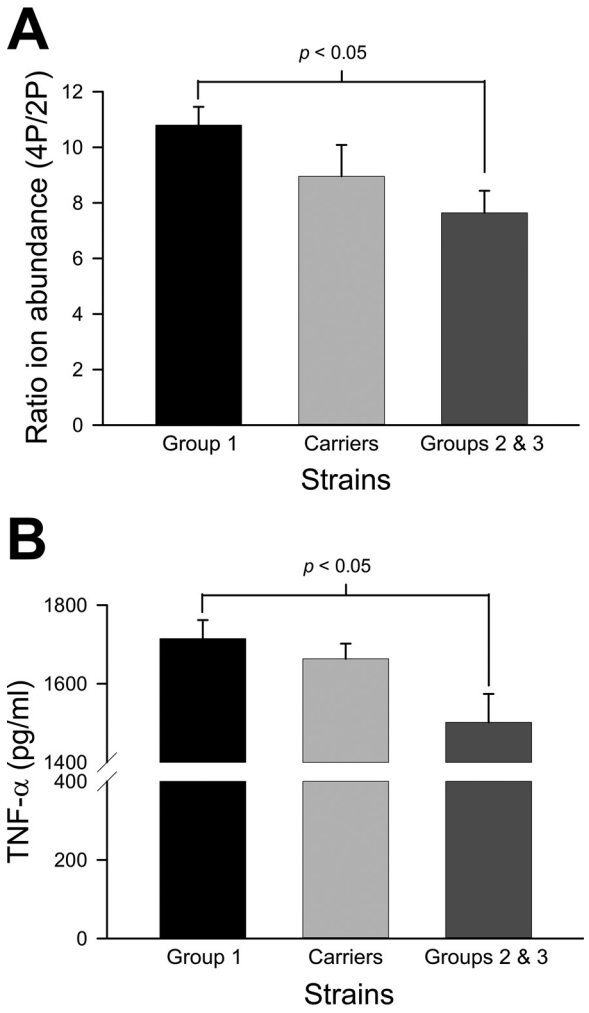


FIGURE 4. *A, graph* presents the ratio of the ion abundance of prompt fragment ion peaks for lipid A moieties with four phosphoryl moieties (3P and 1 PEA) relative to the abundance of peaks for lipid A moieties with two phosphoryl moieties for three different groups. Group 1 represents invasive strains isolated from 19 patients who had meningitis; group 2 LOS is from 12 patients had septicemia, and group 3 was from eight patients who had both septicemia and meningitis. LOS was isolated from bacteria isolated from 25 individuals who carried *N. meningitidis* but did not have any disease. There was significantly less phosphorylation of the lipid A in groups 2 and 3 compared with group 1 ($p < 0.05$). *B, graph* shows the levels of TNF-α induced in THP-1 human monocytes by 18-h treatment with 100 ng/ml LOS from 11 strains of group 1, 18 strains of groups 2 and 3, and 18 strains from carriers. The TNF-α induction by LOS from bacteria in group 1 was significantly greater than that of LOS from bacteria from groups 2 and 3 ($p < 0.05$).

found that decreases in the degree of phosphorylation, phosphoethanolaminylation, and *in vitro* inflammatory activity of the lipid A from invasive isolates of *N. meningitidis* were correlated with increased occurrence of septicemia in patient diagnostic groups 2 and 3. Isolates from group 1 that did not cause septicemia expressed more highly inflammatory and highly phosphorylated lipid A. Thus, the most highly phosphorylated bacterial isolates had relatively diminished ability to survive systemically. Our results show that the pathogenicity of the *N. meningitidis* isolates expressing the most highly inflammatory lipid A was curtailed in terms of ability to cause systemic disease in comparison with *N. meningitidis* strains in which the lipid A was less highly phosphorylated and caused more systemic infections. Our data suggest that differences in phosphorylation of the lipid A play a role in the relative ability of the bacteria to infect cells and survive in the periphery or in the CNS or that differences in lipid A phosphorylation reflect or modulate other differences between the strains that affect the relative fitness of the bacteria to invade and survive in these compartments.

TABLE 4
Expression of PEA (A), *O*-acetate (B), and sialic acid (C) on OS

A

Disease Group	0	1	2	Significance
1	3	13	3	---
2[a]	0	9	2	NS
3[a]	0	7	1	NS
All	3	30	7	---
Carriers[b]	9	15	1	$p = 0.010$

B

Disease Group	n	+ *O*-acetate	Significance
1	19	11	---
2[a]	12	6	NS
3[a]	8	6	NS
All	40	24	---
Carriers[b]	25	24	$p = 0.0035$

C

Disease Group	n	+ Sialic acid	Significance
All	40	37	---
Carriers	25	18	$p = 0.0365$

[a] Data were compared to group 1.
[b] Data were compared to all disease groups.

Although we have a significant understanding of the identity of the various genes needed to synthesize LOS, how the various proteins interact during the biosynthetic process is not understood. The prevalence of a subset of alleles of the LOS biosynthetic genes from meningococcal strains with different colonization outcomes suggests that when the host is unable to restrict colonization to the carrier state, there is an optimal LOS structure that enhances the chance of disease. This structure may not be entirely predictable based on the presence or absence of a set of genes, because the final structure is the product of numerous enzymes and substrates. Also as we have shown with phosphorylation, the degree of substitution in addition to the presence or complete absence of a substituent on the LOS of a strain can affect bioactivity. Our structural studies indicate that fine structural diversity can tip the balance in favor of the pathogen, and our genomic analysis of the genes involved in LOS biosynthesis supports our overall hypothesis that certain LOS structural modifications correlate with specific outcomes of colonization. However, the overall conclusion regarding the genomic analysis could be biased by the composition of the database used because although the PubMLST database contains >32,000 genomes, the presence of numerous sequences from a small number of outbreaks has the potential to skew the data. Nonetheless, the strains in this study reflected the allelic diversity of these genes in disease and carrier isolates from the larger genomic database. We intend to perform additional bioinformatic analyses of these genes to further confirm our findings.

Our previous studies of neisserial LOS and innate immunity have shown that the degree of phosphorylation of the lipid A component can be correlated with the inflammatory potential

TABLE 5

Genomic diversity of LOS biosynthetic genes in *N. meningitidis*

Total number of strains in each column is as follows: unique alleles all strains (32,202), carrier (6596), meningitis (3252), septicemia (958), meningitis and septicemia (669), and invasive not specified (16,686).

Locus[a]	Unique alleles	Carrier	Meningitis	Septicemia	Meningitis and septicemia	Invasive not specified
ADP-heptose biosynthesis						
NEIS0769 (*hldA*)	108	30	6	2	2	86
NEIS0773 (*hldD*)	145	42	14	3	4	115
NEIS2014 (*gmhB*)	85	31	9	3	5	65
NEIS2055 (*hldC*)	82	30	10	3	4	69
NEIS2070 (*gmhA*)	99	42	9	3	4	74
Kdo addition						
NEIS0624 (*kdsB*)	175	46	13	3	5	142
NEIS1218 (*kdsA*)	105	35	10	2	6	82
NEIS1815 (*kdsC*)	80	27	8	6	6	64
NEIS1816 (*kpsF*)	206	53	10	5	6	162
NEIS2152 (*kdtA*)	441	94	17	6	6	330
Lipid A biosynthesis, acyltransferases						
NEIS0168 (*lpxA*)	135	46	15	5	6	104
NEIS0171 (*lpxD*)	216	52	15	6	6	166
NEIS0421 (*lpxL2*)	126	31	11	6	5	105
NEIS1351 (*lpxL*)	201	50	14	4	5	159
Lipid A biosynthesis, other						
NEIS0001 (*lpxC*)	416	100	17	5	5	324
NEIS0191 (*lpxB*)	190	52	15	5	8	150
NEIS0483 (*lpxH*)	174	49	14	7	8	142
NEIS0621 (*lpxK*)	158	47	17	3	5	115
NEIS1553 (*lptA*)	353	93	24	9	10	272
LOS α chain transferases						
NEIS0899 (*lst*)	281	53	15	7	7	224
NEIS1618 (*lgtF*)	141	47	14	5	6	103
NEIS1900 (*lgtE*)	360	75	31	13	5	285
NEIS1901 (*lgtB*)	347	62	27	12	6	270
NEIS1902 (*lgtA*)	191	47	13	8	8	151
NEIS2154 (*lgtC*)	26	8	5	1	0	22
NEIS2155 (*lgtD*)	254	64	18	8	9	203
LOS inner core transferases						
NEIS0291 (*lot*)	262	62	17	5	5	203
NEIS1456 (*rfaF*)	230	72	16	7	7	169
NEIS1619 (*rfaK*)	184	53	11	5	6	151
NEIS1986 (*lpt3*)	228	52	14	5	6	183
NEIS2011 (*lgtG*)	80	26	11	3	6	62
NEIS2012 (*lpt6*)	59	22	10	4	5	44
NEIS2134 (*rfaC*)	251	55	23	7	6	196
LOS transport/export						
NEIS0304 (*msbA*)	281	71	15	8	8	210
NEIS0657 (*rlpB*)	66	18	6	2	5	45
NEIS1812 (*yhbG*)	147	37	10	5	4	116
NEIS1813 (*ostA*)	94	26	4	2	1	71
NEIS1814 (*lptC*)	114	38	9	4	4	85
UDP-GlcNAc synthesis						
NEIS0015 (*glmU*)	323	81	18	6	8	250
UDP-glucose and UDP-galactose biosynthesis						
NEIS0048 (*galE*)	236	49	14	6	3	182

[a] Locus definitions are organized according to various steps in the biosynthetic process.

of the LOS as revealed by cytokine induction in human monocytes and, in general, with the severity of neisserial infections. For example, among the eight commensal *Neisseria* species that rarely cause disease, *N. cinerea*, *N. elongata*, *N. flavescens*, *N. lactamica*, *N. mucosa*, *N. polysaccharea*, *N. sicca*, and *N. subflava*, we found that only two species, *N. lactamica* and *N. elongata*, express functional *lptA*, the enzyme that transfers PEA to the lipid A (3). We also found that the inflammatory potential of LOS made by commensal or *lptA* knock-out *Neisseria* not expressing PEA on the lipid A was significantly reduced. *N. gonorrhoeae* produces predominantly a localized, mild mucosal infection that rarely disseminates or induces protective immunity unlike *N. meningitidis*, and accordingly the lipid A of gonococcal LOS is significantly less phosphorylated and less inflammatory than meningococcal LOS (2, 10).

Recent analysis of experimental infections with mixtures of wild-type *N. gonorrhoeae* and isogenic mutants that lacked *lptA* in both mice and men showed that *lptA* expression conferred a significant increase in the ability of the bacteria to survive in mammalian hosts (40). Modification of lipid A with PEA has been found to increase the resistance of *N. gonorrhoeae* to complement killing in normal human serum by increasing the binding of the complement regulatory protein C4b-binding protein and increasing resistance to endogenous cationic antimicrobial peptides (41, 42). In *N. meningitidis*, modification of lipid A with PEA has been shown to inhibit bactericidal activity of cathepsin G within neutrophil extracellular traps (4) and to increase the adhesion of the bacteria to human cells (5).

Our laboratory previously reported that in addition to greater inflammatory potential, neisserial LOS with more

TABLE 6
Allelic diversity of *lst* and *lptA*

Carrier[a]		Meningitis		Septicemia		Meningitis and septicemia		Invasive not specified	
Allele no.[b]	No. of isolates[c]	Allele no.[b]	No. of isolates[c]	Allele no.[b]	No. of isolates[c]	Allele no.[b]	No. of isolates[c]	Allele no.[b]	No. of isolates[c]
lst									
1	67	1	95	150	20	150	13	1	912
3	64	201	15	1	17	1	4	16	611
15	48	150	6	25	2	2	1	18	369
199	17	3	5	8	1	3	1	241	257
16	15	8	4	48	1	4	1	25	173
18	14	25	4	95	1	18	1	2	158
266	11	18	3	202	1	25	1	111	133
43	9	202	2					15	125
25	6	214	2					3	77
12	5	4	1					90	60
412	5	43	1					150	57
4	4	49	1					108	55
94	3	264	1					32	46
268	3	287	1					49	39
13	2	298	1					4	36
lptA									
6	60	45	79	9	19	136	11	45	547
9	47	9	24	45	9	9	6	11	496
13	19	334	12	136	7	1	1	9	393
110	19	6	6	183	5	2	1	80	337
364	17	38	6	11	1	3	1	94	177
89	14	11	4	34	1	6	1	1	159
54	11	136	4	38	1	45	1	34	104
45	10	183	3	88	1	130	1	6	95
513	10	337	2	96	1	183	1	53	87
15	9	387	2			369	1	89	81
18	9	2	1					136	78
85	8	13	1					18	69
113	6	14	1					96	68
516	6	28	1					93	53
17	4	61	1					54	40

[a] Column labels indicate the disease state from which the isolate was obtained.
[b] Allele number is the identifier provided by PubMLST.org.
[c] Number of isolates represents the number of times the DNA sequence encoding that allele was identified in the sequence database.

highly phosphorylated lipid A also has greater ability to induce immune tolerance *in vitro*. Dephosphorylation of the LOS abrogated its tolerogenic activity (27). We showed that the more highly phosphorylated LOS, which caused more toll-like receptor (TLR) 4-mediated signaling, had greater capacity for up-regulation of the expression microRNA-146a (miR-146a) that led to the reduced expression of p38, IL-1R-associated kinase 1, TNF receptor-associated factor 6, and TLR4 signaling. Transfection of cells with an inhibitor and a mimic of miR-146a confirmed its role in tolerance induction. Our data acquired with neisserial LOS are in accord with those of other laboratories that have reported the critical role of miR-146a in endotoxin tolerance (43, 44).

Overall, it has been increasingly recognized that the suppression of immunity is a component of the response to Gram-negative bacterial septicemia that facilitates infection and leads to a subsequent systemic response and circulatory collapse that is the primary cause of death in industrialized countries. Supporting this concept, genome-wide expression profiling revealed that pathogenic *N. meningitidis* can suppress host responses (45). Significant evidence indicates that expression of highly phosphorylated, hexaacylated LOS that leads to homeostatic signaling in response to inflammation mediated by NF-κB induction by the TIR domain-containing adaptor inducing IFN-β (TRIF)/TRIF-related adaptor molecule (TRAM) pathway is critical to the ability of *N. meningitidis* to induce immune tolerance, evade the host immune system, and cause disease (27, 46, 47). Importantly, the immune responses in the systemic circulation and the brain have been shown to differ as injection of LOS from *N. meningitidis* systemically was reported to induce tolerance, whereas injection of the LOS in the subarachnoid space of rabbits did not (46).

There are endogenous enzymes, alkaline phosphatases and acyloxyacyl hydrolase (AOAH), that act on lipid A to hydrolyze the phosphate moieties and *O*-linked acyl groups on lipid A, respectively. The cleavage of phosphates on lipid A by alkaline phosphatases reduces inflammation induced by LPS. Because of its ability to detoxify LPS, the therapeutic potential of alkaline phosphatase treatment has been tested and has shown promising results in both animal and human studies of septicemia (48–51).

There are data indicating that expression of AOAH can be induced by TLR4 signaling (52). Thus, systemic infections by *N. meningitidis* could have been preferentially enabled by differential expression of AOAH induced by the less highly phosphorylated, less tolerogenic LOS that caused septicemia in isolates from groups 2 and 3. Greater TLR4 signaling by the LOS from group 2 and 3 strains leading to the induction of AOAH and hydrolysis of the *O*-linked acyl groups on the lipid A of the LOS could have dampened both innate and adaptive immune responses facilitating systemic infection.

AOAH cleaves the two lauryl groups linked to the C3-OH of the *N*-linked myristoyl moieties on neisserial lipid A to produce an LOS with tetraacylated lipid A. Tetraacylated lipid A is not inflammatory in humans but binds to TLR4 and thus acts as a competitive inhibitor of TLR4 signaling (53–56). Tetraacyla-

tion of the lipid A is not sufficient to maintain the conformational shape of most of the larger OS epitopes of the neisserial LOS to enable binding of LOS-specific antibody (57, 58). We have shown that human bactericidal IgG antibodies that bind LOS of *N. meningitidis* and mediate complement-dependent bactericidal activity recognize an internal lacto-*N*-neotetraose structure on the LOS (8). Thus, we postulate that increased expression of AOAH due to TLR4-mediated signal transduction induced by the LOS from groups 2 and 3 led to more deacylation of the neisserial LOS, reducing TLR4 signaling and curtailing innate immune responses, and also limiting adaptive immune responses by reducing recognition of the LOS by bactericidal antibodies (6, 8, 59, 60).

We propose that there was comparatively more efficacious induction of TLR4 by the more highly phosphorylated LOS from group 1 isolates, leading to the suppression of TLR4 signaling due to downstream induction of higher level expression of miR-146a. Reduction of TLR4 signaling by miR-146a should have limited the induction of AOAH expression and the AOAH-mediated reduction of TLR4 signaling and adaptive immunity to LOS. Thus, we hypothesize that the LOS-specific adaptive immune response was more vigorous toward strains with the highly phosphorylated LOS leading to more antibody-mediated bactericidal and opsonophagocytic bacterial killing and less systemic infectivity.

There is strong evidence, including *in vitro* studies in sera of individuals deficient in components of the complement system, that alternative and the classical pathways and bactericidal antibody play important roles in *N. meningitidis* infections (60–63). Adaptive immune responses are muted in the brain compared with the bloodstream, and this could explain the fact that the ability of strains with more highly phosphorylated LOS to infect the brain was undiminished compared with strains with less phosphoryl substitution.

Our postulated mechanisms fit the results we have obtained that show that strains producing LOS with less highly phosphorylated lipid A were better able to cause systemic meningococcal infections that are more deadly than infections limited to the CNS (64). In addition, our model is in accordance with several aspects of the clinical progression of *N. meningitidis* infections. Despite the fulminant nature of meningococcal infections that can lead to death within a few hours after the first onset of symptoms, the time from exposure to *N. meningitidis* to disease is thought to be 7–10 days, which would enable variable production of meningococcus-specific antibody to play a role in the disease (65, 66). Clinical observations have divided meningococcal infections into two groups based on the time elapsed between the first observation of symptoms and hospital admission. In three studies, the median times from symptomatic onset to admission for patients with fatal septicemia were less than half the time observed for patients with only meningitis (64, 67). These data are consistent with our model in which the invasive isolates fell into two categories. Those isolates that were more able to suppress robust inflammation induced by TLR4 signaling initially were associated with CNS infections that are less deadly than systemic infections.

Although *N. meningitidis* is carried in the nasopharynx by ~8–20% of the population, the bacteria results in disease characterized by meningitis and septicemia only at low frequencies. Nonetheless, asymptomatic carriage of *N. meningitidis* is of concern as it is the mechanism by which the reservoir of endemic meningococci is maintained within the population. Similarly, asymptomatic gonococcal infection of women plays an important role in maintaining transmission of *N. gonorrhoeae* (68). Neisserial species other than meningococci also colonize the upper respiratory tract but are classified as part of the normal flora. Although scattered reports of infections by commensal *Neisseria* exist (69), the organisms are regarded as non-pathogenic. However, despite the importance of the carrier and asymptomatic state in *Neisseria* biology, the relationships between carriage and disease have not been fully explored. In particular, little is known regarding the innate immune responses elicited by asymptomatic carriage (70).

Six of the carrier strains out of a total of 25 expressed a pentaacylated lipid A, whereas all 40 LOS from invasive strains analyzed expressed hexaacylated lipid A. All of the strains with pentaacylated lipid A were of serotype Y. Mutations in *lpxL1* resulting in the expression of pentaacylated lipid A by invasive strains of *N. meningitidis* have previously been reported (71, 72) and were reported to occur in more than 5% of 448 invasive strains isolated in the Netherlands (73). *lpxL1* mutant strains were also identified in Y serogroup disease isolates from South Africa and England (74, 75). Our results differ in that *lpxL1* mutants were detected exclusively in carrier and not in invasive strains of *N. meningitidis*.

We found that there were no statistically significant differences in the degree of phosphorylation of the lipid A of the carrier strains compared with the disease strains as a whole or compared with group 1 or groups 2 and 3. This result was in accord with the lack of difference in the inflammatory signaling induced by the LOS of the hexaacylated carrier strains compared with the disease strains.

An examination of the OS moieties of the LOS of carrier strains showed significant variation in the number of sialic acid and PEA groups that were decreased and *O*-acetate moieties that were increased, in comparison with LOS of invasive stains. We and others have shown that sialylation of meningococcal and gonococcal LOS expressing the common lacto-*N*-neotetraose phenotype increases serum resistance by preventing the binding of serum IgG to antigenic regions on lacto-*N*-neotetraose, thereby inhibiting killing by complement and by neutrophils (8, 60, 76, 77). Recent data show that the alternative complement pathway is inhibited interdependently by meningococcal expression of sialylated LOS, some of the capsule serogroups, the neisserial surface protein A, and factor H-binding protein. Sialylation of the lacto-*N*-neotetraose portion of LOS subverts complement-mediated bactericidal activity by binding host factor H, which is a regulatory protein of the alternative complement pathway (78). Illustrating its significance to infection, human factor H transgenic rats that were infected with *N. meningitidis* all developed bacteremia, whereas no bacteremia was seen in non-transgenic rats (79).

The *O*-acetylation of the γ-GlcNAc of meningococcal LOS has been postulated to sterically inhibit phosphoethanolaminylation of HepII *O*-3 by Lpt3 (25). This postulate is supported by our data showing that LOS from 96% of carrier strains was

O-acetylated, whereas only 60% of disease-causing strains expressed *O*-acetate, and that overall the OS of the carrier strains had significantly less substitution with PEA. The presence of more *O*-3 PEA groups on HepII of the LOS would increase resistance to killing of invasive strains during bloodstream dissemination due to inhibition of binding to complement factor C4b. PEA on HepII in LOS from *N. meningitidis* can form amide bonds with the classical pathway component C4b. Strains expressing LOS with *O*-3-linked PEA were less efficient in binding C4b than strains expressing LOS with PEA on *O*-6, and strains of *N. meningitidis* expressing more *O*-6 PEA were more susceptible to complement-mediated killing in serum bactericidal assays (6). Expression of PEA on the *O*-3 of HepII has been reported to be a distinguishing feature of the majority of *N. meningitidis* strains that cause disease (6).

Our analyses of the LOS of invasive strains of *N. meningitidis* show that there is a complex relationship between the extent of lipid A substitution with phosphate and PEA groups and the relative ability to survive in the bloodstream or in the brain and cause disease. The data suggest that meningococci have evolved to exploit various homeostatic aspects of innate immunity. The decreased sialylation and phosphoethanolaminylation and increased *O*-acetylation of the OS portion of the LOS from carrier strains compared with invasive strains indicate that the presence of these structural features are likely important in the ability of the disease-causing bacteria to evade recognition by LOS-specific bactericidal IgG and complement-mediated lysis. Reduced sialylation and phosphoethanolaminylation would lessen pathogenicity of the carrier isolates. Our findings indicate that sialylation and phosphoethanolaminylation of the OS along with the phosphorylation and acylation of the lipid A play significant roles in the pathophysiology of infection and highlight the complex adaptation of the meningococcus to its uniquely human host.

Author Contributions—C. M. J. and G. A. J. designed the study, analyzed data, and co-wrote the manuscript. C. M. J. and N. J. P. performed LOS mass spectrometry and analyzed the data. E. R. chose the meningococcal strains from the Norwegian vaccine trial strain collection and was involved in study design. E. A. H. made clinical diagnoses and collected strains during the Norwegian vaccine trial. D. C. S. performed the bioinformatic and genomic analyses and wrote portions of the manuscript. R. D. provided technical assistance in purification of LOS. M. L. performed and analyzed the experiments shown in Fig. 4*B*. All authors reviewed the results and approved the final version of the manuscript.

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